Monitoring Long-term Stress through Hair Cortisol Analysis for Conservation and Captive Management in Chimpanzees (*Pan troglodytes*) and Orangutans (*Pongo* spp.)

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I Abstract

Increased long-term stress is known to severely reduce animal fitness and to have detrimental effects on population size if it affects whole groups. Wild orangutans (*Pongo sp.*), chimpanzees (*Pan troglodytes*) and other great ape species face a wide variety of anthropogenic impacts in their natural habitats. These are often suspected of causing increased long-term stress in the animals. Unfortunately, monitoring programs trying to assess the severity of potential stressors are rare despite their importance for informed decisions in conservation. At the same time, zoos face increasing ethical concerns about the confinement of large animals in general and great apes in particular. Similar to the situation in wild animals, practical monitoring protocols that guarantee welfare in zoo living animals, are still pending. These deficits in the monitoring of wild and captive great apes may in part be due to the lack of cost-effective monitoring tools.

In this thesis, I therefore set out to test whether cortisol concentrations measured in hair (HCC) reliably reflect long-term stress in orangutans and chimpanzees in wild and captive environments. Aiming at a better understanding of this comparatively novel method, I first investigated potentially confounding effects. Here I found significant differences in absolute HCC among various body regions, possibly driven by differences in skin blood flow. Yet HCC among various body regions was driven by one common factor and provided similar biological meaning. Thus, a random mixture of hair from different body regions, such as the naturally shed hair found in sleeping nests from great apes, can be used at the cost of a lower signal to noise ratio. Concerning HCC stability along the hair shaft, I presented evidence that HCC decreased towards distal segments of the hair if animals were exposed to ambient weather conditions. However, importantly, the rank order was retained across all segments. Thus, the investigated length of hair should be kept constant for inter-individual comparisons.

Second, concerning the biological validation, I found that HCC measured along the extended hair of orangutans reflects periods of increased stress levels for up to several years. In a group comparison, orangutans with perceived long-term stress exhibited significantly higher HCC. For chimpanzees, I found that HCC in a group of 36 chimpanzees correlated highly with the subjective estimates of stress provided by animal keepers. Applying HCC analysis to wild chimpanzees allowed me to assess the effects of anthropogenic impacts in habituated animals, chimpanzees that are

used to being followed by human observers, and for the first time unhabituated animals. Analyses of nest-hair HCC in four chimpanzee communities in western Uganda did not reveal elevated stress levels due to ecotourism compared to a control group with no human contact. In contrast, a chimpanzee community living in a small forest fragment experiencing regular aggressive conflicts with humans exhibited significantly increased HCC, indicating increased stress levels.

In conclusion, my results suggest that hair cortisol analysis is a powerful tool that can help understanding the impact of anthropogenic disturbances on wild chimpanzees and other great apes as well as it could be used for monitoring in captive contexts.

II Zusammenfassung

Es ist bekannt, dass langfristig erhöhter Stress die Fitness von Tieren stark beeinträchtigen kann. Betrifft dies nicht nur einzelne Tiere sondern ganze Gruppen so kann Langzeitstress verheerende Auswirkungen auf ganze Populationen haben und zu lokalem Aussterben führen. Orang-Utans (*Pongo sp.*) und Schimpansen (*Pan troglodytes*) begegnen heute zahlreichen anthropogenen Einflüssen, die im Verdacht stehen, Langzeitstress bei den Tieren zu verursachen. Untersuchungen, die den Effekt potentieller Stressoren feststellen, sind trotz ihrer Relevanz für einen fundierten Tierschutz allerdings selten. Gleichzeitig müssen sich Zoos zunehmend mit moralischen Bedenken gegenüber der Haltung großer Tiere im Allgemeinen und der Haltung von Menschenaffen im Besonderen auseinandersetzen. Trotz dieser Entwicklungen gibt es für Zoos bis heute keine Überwachungsprotokolle, die das Wohlergehen der Tiere garantieren. Das Fehlen solcher Untersuchungen bei freilebenden und zoolebenden Tieren ergibt sich zum Teil daraus, dass praktikable Messmethoden bislang fehlten.

Während der folgenden Arbeit untersuchte ich daher, ob die im Haar gemessenen Cortisolkonzentrationen (HCC) zuverlässig das mittlere Stressniveau von Schimpansen und Orang-Utans reflektieren. Da die Methode der Cortisolmessung im Haar noch recht neu ist, konzentrierte ich mich zunächst auf Untersuchungen, die ein besseres Verständnis für potentielle Einflussfaktoren ermöglichen sollten. In diesem Zusammenhang zeigte sich, dass die mittleren HCC in den verschiedenen Körperstellen variieren, was sich möglicherweise durch Unterschiede in der Hautdurchblutung erklären lässt. Gleichzeitig konnte ich zeigen, dass die Varianz von HCC einem gemeinsamen treibenden Faktor zugrundeliegt und die HCC in allen Körperstellen somit die gleichen biologischen Inhalte reflektieren. Dieser Befund erlaubt es auch, dass eine zufällige Mischung von Haaren verschiedener Körperstellen, wie man sie in den Nestern von Menschenaffen findet, für die Analyse von Haarcortisol genutzt werden kann, auch wenn man dabei ein niedrigeres Signal-Rausch-Verhältnis in Kauf nehmen muss. Bei der Betrachtung der HCC-Stabilität entlang des Haarschafts zeigte ich erstmals für Tiere, dass die HCC zum distalen Ende hin, vermutlich durch umgebende Witterungsbedingungen, abnehmen, dass jedoch die Rangreihenfolge in den verschiedenen Segmenten entlang des Haarschaftes bestehen bleibt. Demzufolge sollte die Haarlänge bei Untersuchungen mit mehreren Tieren immer konstant gehalten werden.

Während der biologischen Validierung konnte ich zeigen, dass Perioden mit erhöhtem Stressniveau durch die HCC entlang der sehr langen Haare von Orang-Utans abgebildet und über mehrere Jahre hinweg gemessen werden können. Zusätzlich wiesen stark gestresste Orang-Utans in einem Gruppenvergleich signifikant höhere HCC auf als solche, die den Tierpflegern zufolge keinem erhöhten Langzeit-Stress ausgesetzt waren. Anhand einer Gruppe von 36 Schimpansen konnte ich zeigen, dass die HCC hochsignifikant mit den Stresseinschätzungen von Tierpflegern korrelieren. Mithilfe der HCC-Analyse konnte ich schließlich auch untersuchen, inwieweit anthropogene Einflüsse freilebende Schimpansen beeinträchtigen. Hier untersuchte ich insgesamt vier Schimpansengruppen habituierter, also an den Menschen gewöhnter, und nicht-habituierter Tiere im Westen Ugandas. Dabei fand sich kein Hinweis darauf, dass die für den Ökotourismus im Budongo Waldreservat habituierten Schimpansen gestresster wären als die nicht-habituierte Nachbargruppe. Im Gegensatz dazu zeigten die Schimpansen signifikant erhöhte HCC, die in einem kleinen Waldfragment lebten und regelmäßig negativen Auseinandersetzungen mit der lokalen Bevölkerung ausgesetzt waren.

Zusammenfassend zeigen meine Ergebnisse, dass die Analyse von Haarcortisol das Potenzial hat, die Auswirkung menschlicher Einflüsse auf freilebende Schimpansen und anderer Menschenaffen einzuschätzen, genauso wie die Methode im Zusammenhang mit dem Management von Zootieren effektiv eingesetzt werden kann.

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1. Introduction

1.1 The need to monitor animal well-being in wild and captive environments

One of the major threats for wild great apes is habitat degradation and fragmentation, which has led to a steep decline in chimpanzee and orangutan population size and distribution as well as increased interactions with humans. Consequently, today many great ape populations face anthropogenic impacts of various kinds and questions about how well and under which circumstances they can cope with human influence is of central importance for conservation programs. An increasing number of studies have been conducted investigating the effects anthropogenic impacts have had on non-human primates, with varying results: Howler monkeys and spider monkeys , for example, were found to show increased stress levels in the context of forest fragmentation (Behie et al., 2010; Martínez-Mota et al., 2007; Rangel-Negrín et al., 2014, Rimbach et al., 2013b) and high human presence (Vanlangendonck et al., 2015). Yet, other studies of the same species found no influence of human presence and fragmentation (Rimbach et al., 2013; Vanlangendonck et al., 2015). Because this kind of anthropogenic disturbance on primates is bound to show a dramatic increase in the coming decades, these ambiguous results illustrate that more research is urgently needed in order to understand its impact.

Ecotourism is one of the contexts in which it would be important to know the effect humans have on the animals. Ecotourism is defined as responsible travel to natural areas that conserves the environment, sustains the well-being of the local people, and involves interpretation and education (TIES, 2015). It is often considered a useful conservation tool because it protects the habitat at the same time as it promotes the local economy and raises awareness (Hvenegaard, 2014). However, an increasing number of studies on various species suggest that tourism can have adverse effects on the animals that are to be protected, in the form of reduced breeding success (Ellenberg et al., 2007; Kerbiriou et al., 2009), decreased feeding times due to increased vigilance (Duchesne et al., 2000), increased risk of pathogen transmission (Muehlenbein and Wallis, 2014), and generally increased stress levels (howler monkeys: Behie et al., 2010; Rangel-Negrín et al., 2014, barbary macaques: Maréchal et al., 2011, western lowland gorillas: Shutt et al., 2014, in contrast see Aguilar-Melo et al., 2013; Muehlenbein et al., 2012). Unfortunately, guidelines for

"good ecotourism" are usually not backed by scientific results and monitoring programs trying to identify influential factors are absent in most tourism sites.

Another context in which objective estimates about the well-being of animals would be highly advantageous is in captive management. Increasing ethical concerns about the confinement of animals have led to calls for higher and uniform standards for farm and zoo animals. Whereas previous concerns about captive animals mainly focused on health and productivity of farm animals, attention has recently shifted towards more ethical considerations about their confinement (Hewson, 2003). This has led, for example, to a change in the German constitution, where in 2002 a law for the protection of animals was implemented (Art. 20a GG), stating that human needs have to be balanced with animal welfare. As a consequence, animal welfare science has made important progress leading to the development of practical protocols for the assessment and improvement of welfare in farm animals (Webster et al., 2004; Webster, 2005). Yet, a similar program for zoo animals is still pending. This is all the more surprising as many animal rights activists campaign for the abolition of confinement for certain species, especially great apes. Nevertheless, an innovative approach to assess animal welfare on an individual level recently proposed the use of regular animal keeper-based questionnaires, including scores on technical details such as health, aswell as on emotional states such as joy and anger (Whitham and Wielebnowski, 2013, 2009). However, to date the question remains whether this approach will be able to objectively reflect between-group differences in order to judge the quality of different holding facilities. In the absence of external validation, the validity of animal keepers' scores may potentially be restricted to their own animals, and some might argue even these measures are too subjective. An attempt to objectively measure animal well-being in zoos was made by Weingrill et al. (2011) assessing the average animal stress level through repeated measures of glucocorticoid stress markers in feces. While long-term physiological measures will not depict the whole scope of animal welfare, they can still serve as an objective indicator of animal well-being.

1.2 Long-term stress levels indicate animal welfare and fitness

In a general concept of stress, any challenging or demanding physiological or psychological parameter can become a stressor (Selye, 1950) due to its potential to affect homeostasis (Chrousos, 1998). In an attempt to elaborate this concept and

especially to clarify the terminology, McEwen and Wingfield (2003) suggested the *concept of allostasis*. According to this concept, any response to a stressor places the organism in an *allostatic state* which is defined as a state of physiological imbalance or change in order to re-establish *homeostasis* (physiological balance). Yet, this allostatic state, that influences a wide variety of physiological pathways, is costly for the organism. These costs are defined as the *allostatic load* and may eventually lead to *allostatic overload* resulting in the organism's break down.

From a physiological point of view, within seconds following a stressor, the sympathetic nervous system mediates the secretion of epinephrine (adrenaline) which immediately mobilizes energy in order to allow the organism to "fight or flight" (Cannon, 1916, 1929). This immediate stress response is followed by the activation of the hypothalamic-pituitary-adrenal (HPA) axis a few minutes later, resulting in the secretion of glucocorticoid hormones (GC), mainly cortisol in large mammals including all primates and corticosterone in small mammals, birds, fish, and reptiles (Nelson, 2011). GC contributes to the increase in immediately availability of energy through increased blood sugar concentration via gluconeogenesis and the metabolism of fat and proteins. As a trade-off, energetically expensive pathways not needed for the immediate survival are suppressed or down regulated, like those pathways related to the immune response, reproduction and growth (Nelson, 2011).

Consequently, the stress response mechanism with increased GC levels is adaptive to cope with acute stressors, e.g. running from a predator or coping with short-term environmental perturbations. Unfortunately, the same mechanism is maladaptive if stressors are persistent. Without sufficient time to recover, a prolonged stress response reduces animal fitness through increased susceptibility to infectious illnesses (Selye, 1950; Sternberg et al., 1992), the dysfunction of various organs (Chrousos, 2009; Chrousos and Gold, 1992), reduced growth (Santos et al., 2000) as well as an increased probability of miscarriages (Arck et al., 2001) or infertility (Cocks, 2007). In addition, it has been suggested that altered stress responses related to chronic stress, can be passed on to the next generation through epigenetic modulation (Franklin et al., 2010). Given these adverse effects are not necessarily restricted to single animals, but act on the population level, long-term stress can have detrimental effects on whole populations, and may even lead to local extinction.

For these reasons, the level of systemic GC secretion is generally seen as a measurable reflection of the allostatic load that an individual perceives (Wingfield,

2013), even though pathologies like depression may alter the stress response of the HPA axis (e.g., Carroll et al., 1976; Yehuda et al., 1996). Thus, measuring long-term GC concentrations can provide important insights into animal well-being on a physiological level.

1.3 Cortisol concentrations in hair facilitate long-term stress monitoring

The amount of systemic cortisol secretion into blood can be measured indirectly and non-invasively in saliva (Kirschbaum and Hellhammer, 1994), urine (Muller and Wrangham, 2004; van Schaik et al., 1991) and feces (Shutt et al., 2012; Weingrill et al., 2011), or invasively in blood plasma (Sapolsky, 1982). However, the glucocorticoid concentration in these matrices only reflects cortisol secretion during a narrow time window of minutes to days. Thus, measuring long-term stress requires repeated sampling of the same individual in order to even out the influence of short-term stressful events as well as that of biological cycles (e.g., circadian rhythm, ovarian cycle of females). In addition, assessment of glucocorticoid metabolites from feces is confounded by metabolite degradation which commences within hours of defecation (Shutt et al., 2012). Hence, regular close proximity to the animals is required, which, in the case of wild animals, necessitates habituation to human observers. However, habituation in chimpanzees, for example, can take up to seven years (Bertolani and Boesch, 2008) and may itself have a negative impact on the animals (Grieser Johns, 1996). Thus, it may prevent the detection of human impacts on the animals. Furthermore, the necessity to cool or dry samples makes sample collection in remote areas very demanding. Thus, while traditionally used methods for physiological stress monitoring are adequate tools for the detection of short-term changes in some species and circumstances, they are often challenging when studying long-term stress levels, especially in wild chimpanzees.

Measuring cortisol concentrations in hair is a promising approach to overcome these problems. Hair can be sampled entirely non-invasively from non-habituated animals through the use of sticky hair traps (or barbed wire) or by collecting it from nests, as in the case of great apes. Over the last decade, the assessment of cortisol in hair has increasingly been valued as an integrated measure of the systemic cortisol secretion over several months (Davenport et al., 2006). Although some uncertainty still exists about the incorporation mechanisms (Figure 1.1), it is widely agreed that cortisol is constantly incorporated into the growing hair shaft (reviewed by

Meyer and Novak, 2012; Russell et al., 2012; Stalder and Kirschbaum, 2012). The notion that HCC reflects the cortisol concentration in blood is supported by an increasing number of indirect validation studies (e.g., Davenport et al., 2006; Dettmer et al., 2012; Kirschbaum et al., 2009) and three studies showing significantly increased HCC in consequence of multiple weekly ACTH injections (del Rosario Gonzalez-de-la-Vara et al., 2011; Mastromonaco et al., 2014; Terwissen et al., 2013).

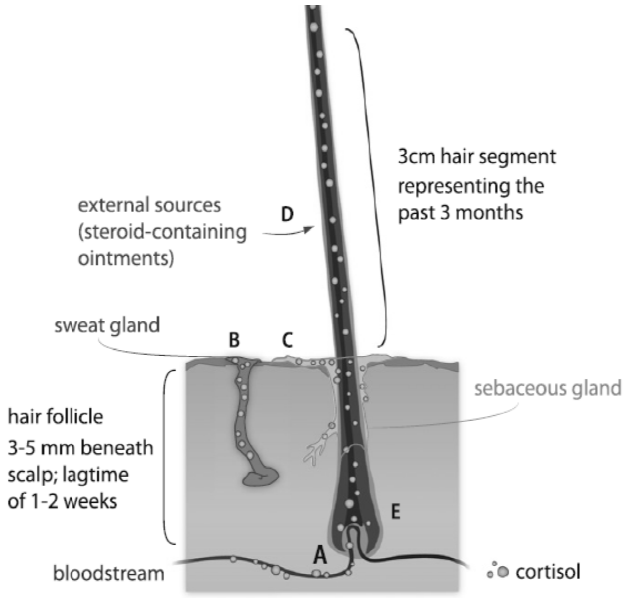

Figure 1.1: Assumed mechanisms of cortisol incorporation into hair and retrospective reflection of long-term cortisol secretion (modified after Russell et al., 2012). Cortisol may be incorporated into hair via passive diffusion from blood (A), sweat (B) and/or sebum (C) as well as from external sources (D). In addition, it has been proposed that locally produced cortisol may also contribute to hair cortisol concentrations (E). (Stalder and Kirschbaum, 2012)

Besides its ability to reflect cortisol levels over long periods in time in one sample, and the added advantage that short-term stressful events and biological rhythms are leveled out, HCC also does not degrade after sampling and samples can therefore be kept for years and shipped without additional cooling or drying (Russell et al., 2012; Webb et al., 2015), which is especially useful in remote study areas with unreliable electricity supply.

1.4 Assessing cortisol concentrations in hair

A relatively simple protocol to measure concentrations of the steroid hormone testosterone in hair has been developed by Koren et al. (2002) and was further modified and validated for the steroid hormone cortisol by Raul et al. (2004) and Davenport et al. (2006). The latter publications especially, inspired the endocrine

laboratory from the research group for Biopsychology at the Technische Universität Dresden run by Prof. Dr. Clemens Kirschbaum. They further demonstrated the biological relevance of hair cortisol concentrations studying pregnant women at different trimesters as biological models because cortisol concentrations are known to differ between various stages of pregnancy (Kirschbaum et al., 2009). In close cooperation with the Kirschbaum group I analyzed all hair samples from this thesis in the biopsychology laboratory, adopting their methodological knowledge and advances from human hair (Gao et al., 2013) to chimpanzee and orangutan hair.

1.5 Variables with the potential to influence hair cortisol concentrations

Numerous laboratories have integrated the assessment of HCC into their laboratory repertoire (e.g., Davenport et al., 2006; Kirschbaum et al., 2009; Koren et al., 2008, 2002; Raul et al., 2004; Sauvé et al., 2007) and a recent round robin test on human hair showed good reproducibility of results among the leading laboratories (Russell et al., 2015). However, whereas the HCC assessment for humans has been standardized with regards to the anatomical site of hair collection and the analyzed length of hair, every laboratory analyzing animal hair has developed its own protocol with regard to body region and length of hair with most animal studies using the whole length of hair (e.g., Fourie et al., 2015). While this may reflect the requirements for different species, it has not been established whether the position along the hair shaft influences HCC and whether all body regions reflect the individual's stress level equally. The latter point is also of core interest when shed hair from sleeping nests of wild great apes is to be used. For these reasons, a large part of this thesis deals with factors that potentially influence HCC.

1.5.1 Waning effect – Cortisol stability along the hair shaft

Although cortisol in hair is stable once samples are stored in an envelope at ambient temperatures, several studies on human hair observed a systematic waning of cortisol towards the distal end of hair (Dettenborn et al., 2010; Gao et al., 2010; Kirschbaum et al., 2009; Skoluda et al., 2012; Steudte et al., 2011, but see Manenschijn et al., 2011; Thomson et al., 2010). Subsequent, *in vitro* experiments by Li and colleagues (2012) showed that the treatment of hair with shampoo solution, warm or hot water only, as well as UV-irradiation, resulted in significant HCC loss. This suggests that cortisol may not only leach from the hair shaft through water but that it may also be degraded through UV-irradiation as has been shown earlier for

cannabinoids in hair (Skopp et al., 2000). For animal hair, a waning effect (HCC decrease) using water was observed only in one *in vitro* experiment where human-like extensive wash-dry procedures resulted in decreased HCC in hair in rhesus macaques (Hamel et al., 2011). However, until now all *in vivo* animal studies showed no systemic HCC difference between the proximal and distal end of hair in both captive animals (Bennett and Hayssen, 2010; Davenport et al., 2006; Yamanashi et al., 2013) and wild grizzly bears (Macbeth et al., 2010). Although none of the *in vivo* animal studies reported a waning effect, the *in vitro* study from Hamel et al. (2011) still raises the question as to whether the hair of some species is resistant to systematic HCC waning along the hair shaft or whether HCC stability is prone to certain ambient conditions, e.g. intense sun or rain.

1.5.2 Body region effect – Cortisol variability among body regions

An increasing number of studies suggest that HCC differs between various body regions (Li et al., 2012; Macbeth et al., 2010; Moya et al., 2013; Terwissen et al., 2013; Yamanashi et al., 2013, but see Comin et al., 2012; Macbeth et al., 2012). At the same time however, it has never been investigated whether HCC from all body regions provides similar biological information although it is fundamental to know whether all body regions are suitable for hair cortisol analysis, whether HCC in all body regions reflects the individual's level of stress equally well and whether hair obtained from different regions provides comparative information. This question is especially critical if one intends to use naturally shed hair in order to work non-invasively with wild great apes. Furthermore, no studies have so far tried to understand the underlying mechanism of this body-region effect. A better causal understanding would help to predict the effect and may further improve our understanding of the cortisol incorporation mechanisms into hair, which are still poorly understood (review: Meyer and Novak, 2012; Russell et al., 2012; Stalder and Kirschbaum, 2012).

1.6 Orangutans *(Pongo sp.)* and chimpanzees (*Pan troglodytes*) as study species

Rapid destructions of suitable habitats for chimpanzees (*Pan troglodytes*) and Sumatran and Bornean orangutans (*Pongo abelii* and *Pongo pygmaeus*) have led to these species being listed as endangered (*Pan troglodytes* and *Pongo pygmaeus*) or critically endangered (*Pongo abelii*) in the "Red List" of the International Union for

Conservation of Nature (Ancrenaz et al., 2008; Oates et al., 2008; Singleton et al., 2008). The consequences are especially dramatic for orangutans where a considerable amount of the animal's habitat is converted into agricultural land which forces the more mobile individuals to transfer into overcrowded habitats resulting in higher death rates and fewer births (Husson et al., 2009, 2002). Alternatively, more philopatric individuals (especially females; van Noordwijk et al., 2012) have nowhere to go and may often be hunted for meat, killed to protect crops or eventually sent to sanctuaries (Husson et al., 2009; Nellemann, 2007). Other orangutans may be kept as pets for years before they are confiscated and sent to sanctuaries. As a consequence, an estimated 2500 of these long-lived orangutans that require caring, have entered sanctuaries during the last two decades (Russon, 2009). For several years, rehabilitation programs have started to reintroduce sanctuary domiciled orangutans back to the wild (Russon, 2009), but only a few small scale monitoring studies have looked at the post-rehabilitation phase through regular behavioral observations (Russon, 2009). These have suggested that most, but not all released animals slowly approach the behavior of wild animals. However, such intense monitoring keeps the animals used to close proximity to humans and cannot be conducted on a large scale.

While the rate of habitat degradation is similarly alarming for chimpanzees, their terrestrial lifestyle may make them behaviorally more flexible and able to adapt to life in human-dominated landscapes (Hockings et al., 2015). For example, several studies have shown that chimpanzees are able to adjust their food preferences and ranging patterns (Hockings, 2009; Hockings et al., 2015, 2012; Hockings and McLennan, 2012; Humle and Matsuzawa, 2004). However, the physiological consequences of these adaptations are unknown although this aspect would provide important insights into the animals' well-being. Similarly, the long-term consequences of ecotourism on chimpanzees are largely unknown. Only few studies have investigated the effects of ecotourism on a behavioral level (Grieser Johns, 1996; Nakamura and Nishida, 2009), or in the point of pathogen transmission (e.g., Zommers et al., 2013). Consequently, the rules for "good ecotourism" for chimpanzees as suggested by Williamson and Macfie (2014) are based on little scientific evidence (Grieser Johns, 1996).

In all these contexts, long-term stress monitoring of wild orangutans and chimpanzees could provide important objective information on the animals' well-being

in order to enable effective management. Hair cortisol analysis would provide a useful method for stress monitoring because the chimpanzees and orangutans, like the other great apes species, build sleeping nests on a daily basis, thus making it possible to collect hair samples from wild animals in an absolutely non-invasive manner. This is particularly useful because, in contrast to smaller primate species, endocrine research on wild orangutans and chimpanzees has been limited to groups and individuals that are used to being followed by human observers in order to collect urine or fecal samples.

Captive management could also greatly benefit from hair cortisol analysis as a cost-effective and objective measure of animal well-being. Against the background of increasing concern regarding the confinement of great apes in zoos, monitoring programs based on hair cortisol analysis could provide important and objective information about the stress level of groups and individuals in terms of different keeping conditions, especially when comparisons with conditions in the wild are possible.

The study of orangutans is especially interesting in the effort to achieve a better understanding of hair cortisol analysis, because their hair usually exceeds 12 cm and can reach up to 50 cm in males. The long hair is particularly suitable for investigations on cortisol stability along the hair shaft. At the same time, I wanted to test whether the long hair of orangutans can be used as a retrospective calendar reporting on the individual's endocrine consequences of stress for more than one year. On the other hand, orangutans are semi-social, and captive groups in zoos usually consist of only few animals that belong to the same family (usually one flanged male and few adult females with their immature offspring). Thus, from a biological perspective (considering sex, age, maturation), groups of captive orangutans are very heterogeneous, making it more difficult to validate the relationship between long-term stress levels and cortisol concentrations in hair.

In contrast, captive and wild chimpanzees live in multi-male-multi-female social systems resulting in bigger groups. Due to the increased number of animals, within- and between-group comparisons are statistically more powerful and facilitate investigations on the effect of biological factors (sex, age) on cortisol concentrations in hair as well as allow validating the relationship between long-term stress and cortisol concentration in hair. However, chimpanzee hair is generally shorter and seldom exceeds 6 to 8 cm. Thus, hair grows for a shorter period in time (anagenic

phase) whereas the time of shedding (telogenic phase) and regeneration of the hair follicle (katagenic phase) are expected to remain constant. For humans, the time between the end of the anagenic phase and the appearance of a new hair is known to last between two and three months for head hairs throughout adult life, (Courtois et al., 1995). Consequently, in shorter hair, neighboring hairs are more likely to be in different phases of the growth cycle and thus represent different time periods, making segmental hair analysis with respect to stress levels in a certain time frame less informative. Nevertheless, analyzing whole hair from chimpanzees might still provide information on the animal's average cortisol concentration which is important for within- and between-group comparisons.

1.7 Aim of the present studies

During this PhD thesis, I aimed at enhancing our understanding of the hair cortisol measurement in chimpanzees and orangutans in order to establish a cost-effective and objective tool for estimation of the animal's well-being in captive and wild environments. Because hair cortisol analysis is still a relatively new method and had not been applied to great apes before, I looked at confounding effects on HCC and examined the response of HCC in various contexts, intra- and inter-individual comparisons and comparisons between groups. After validating HCC measures as a reflection of long-term stress, I investigated the effects of anthropogenic impacts on chimpanzees in order to facilitate conservation programs based on scientifically sound knowledge.

In more detail, I investigated the stability of HCC among body regions and along the hair shaft in European zoo orangutans before examining whether HCC increased in animals that were exposed to severe long-term stressors according to their animal keepers. In addition, I made use of the long hair in orangutans, investigating the possibility of using segmental hair analysis for longitudinal stress records of more than one year (Chapter 2). A homogeneous sample set from semi-wild sanctuary chimpanzees collected from anaesthetized animals during their annual health check as well as from European zoo chimpanzees allowed me to investigate the questions of HCC stability more systematically, whereupon I could also address potential underlying mechanisms of influencing variables (Chapter 3). The same samples from semi-wild chimpanzees served investigations between keeper derived stress estimates and HCC (Chapter 4). Finally, I applied hair cortisol analysis in wild

chimpanzees in order to examine whether group stress levels in chimpanzee communities were influenced by ecotourism and human-wildlife conflicts compared to chimpanzees living in intact forests without adverse human impacts. In addition, I investigated HCC in shed hair from sleeping nests in relation to seasonality and age of nests (Chapter 5).

1.8 Authors contribution

The following chapters have been published (Chapter 2 and 3) or are under revision (Chapter 4 and 5). They were produced in collaboration with the Anthropological Institute and Museum at the University of Zurich (Switzerland), the research group of Biopsychology at the Technische Universität Dresden (Germany), the Chimpanzee Sanctuary and Wildlife Conservation Trust (Uganda), and the Jane Goodall Institute Switzerland.

My advisors Carel P. van Schaik and Clemens Kirschbaum were involved throughout all studies with the study design, the interpretation of the results as well as the writing of the manuscripts. Robert Miller and Tobias Stalder assisted me with the analyses and interpretation of the data as well as with writing the manuscripts. Daniel C. Hänni, Joshua Rukundo and Titus Mukungu helped to collect the samples as well as with the interpretation of the data. Wei Gao performed the hair cortisol analyses with LC-MS/MS and helped writing the manuscripts. I myself was responsible for designing and conducting the following studies, for the collection and the analyses of the hair samples, and I interpreted the data and wrote the manuscripts.

2. Hair as a long-term retrospective cortisol calendar in orangutans (*Pongo spp.*): New perspectives for stress monitoring in captive management and conservation

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2.1 Abstract

This study examined whether the method of hair cortisol analysis is applicable to orangutans (*Pongo spp.*) and can help to advance the objective monitoring of stress in non-human primates. Specifically, we examined whether fundamental prerequisites for hair cortisol analysis are given in orangutans and, subsequently, whether segmental hair analysis may provide a retrospective calendar of long-term cortisol levels. For this, hair samples were examined from 71 zoo-living orangutans (38 males, 33 females, mean age = 23.5 years) for which detailed records of past living conditions were available. Hair samples were cut from defined body regions and were analyzed either in full length or in segments. Results showed that hair cortisol concentrations (HCC) were unrelated to age or sex of the individual animal. HCC were found to be higher in orangutans, with perceived long-term stressful periods (mean HCC = 43.6 ± 26.5 pg/mg, n = 13) compared to animals without perceived stressful periods (19.3 ± 5.5 pg/mg, n = 55, $P < 0.001$). In non-stressed animals, segmental hair analyses revealed that HCC was stable along the hair shaft even when hair reached >40 cm. The possibility of obtaining a retrospective calendar of stress-related cortisol changes through hair analysis was further supported by data of three case studies showing close correspondence between the segmental HCC results and keeper reports of stress exposure during the respective time periods. Finally, low within-animal variation in HCC from different body regions (CV%: 14.3) suggested that this method may also be applicable to naturally shed hair, e.g., as

found in nests of wild orangutans and other great apes. Therefore, using HCC may provide an ideal non-invasive tool for both captive management as well as conservation in orangutans and potentially other great apes.

2.2 Introduction

Until recently, it has been rather difficult to assess the endocrine consequences of chronic stress in animals such as non-human primates. The traditionally used non-invasive cortisol assessment methods in urine (Bahr et al., 2000; Hauser et al., 2008), feces (Weingrill et al., 2011) or saliva (Fuchs et al., 1997) reflect cortisol secretion during a narrow time window. Therefore, measuring long-term hormone levels requires repeated sampling of the same individual in order to even out the influence of short-term stressful events and/or biological cycles. Such a procedure, however, requires animals to be kept in captivity or wild animals to be well habituated to humans which may limit the ecological validity of the respective data. Furthermore, locating wild animals on a regular basis might be difficult in certain species and terrain even if animals are habituated, and habituation is not always desirable because it is time-consuming (Bertolani and Boesch, 2008) and may expose animals to threats of poaching (Morgan and Sanz, 2003).

Measuring cortisol in hair now opens new possibilities for the study of long-term biological consequences of chronic stress exposure. Davenport et al. (2006) was the first to present evidence that the cortisol concentration in hair of rhesus macaques reflects the integrated stress-induced activity of the hypothalamic-pituitary-adrenal axis during hair growth. Since then, hair cortisol measurement has received increasing attention in an ever growing number of fields of application, in both humans (Stalder and Kirschbaum, 2012) and a variety of animal species (e.g., horses: Anielski, 2008; polar bears: Bechshøft et al., 2013, 2011; cows: Burnett, 2014; rabbits: Comin et al., 2012; non-human primates: Fourie et al., 2015b; Fourie and Bernstein, 2011). Besides providing a long-term endocrine record, one key advantage of hair is the stability of HCC under ambient keeping conditions. This enables easy storage and posting of samples (Russell et al., 2012), which could make this method highly valuable in remote places.

Given the continuous growth of hair, an important additional benefit is its potential to derive a retrospective cortisol calendar from segmental hair analyses. However, this is still highly debated in human research. Various studies have suggested that the clinical course of patients with pathological hyper- or hypocortisolism appears to

be well represented in their segmental HCC profile (Manenschijn et al., 2011; Thomson et al., 2010), but other studies have not confirmed this (D'Anna-Hernandez et al., 2011; Kirschbaum et al., 2009). Using hair as a retrospective cortisol calendar requires a number of biological preconditions: First, cortisol incorporation into the hair matrix must be largely completed before the hair reaches the skin surface. Later incorporation (e. g. through sweat) is a potential influence on cortisol levels in human scalp hair (Russell et al., 2012; Skoluda et al., 2012) or ungulate hair (Anielski, 2008; Bullard et al., 1970), but is unlikely to influence HCC in non-human primates because sweat glands are mainly inactive and mostly restricted to their forehead, palms and armpits (Montagna, 1972). Second, there must be stability of HCC over time. Thus, the systematic decrease in cortisol along the hair shaft seen in humans ('washout effect'; e.g., Kirschbaum et al., 2009) should not be observed. Hamel et al. (2011) have shown a decrease in HCC in the hair of rhesus macaques after numerous intense wash/dry procedures using shampoo or water only. Zoo-living animals, however, are not subject to frequent rain and thus a washout effect is unlikely to affect hair of captive animals. Supporting this, no studies have shown cortisol washout effects in animal hair, neither in captive (Davenport et al., 2006) nor free-ranging animals (Macbeth et al., 2010). Third, growth rates of individual hairs within the hair strand should be uniform and the majority of hairs must be in the same growth phase. For cut or pulled-out hair, this means that most hairs should to be in the anagen (active growing) phase for the correlation between segments and time periods to hold. By contrast, naturally shed hairs found in sleeping nests of wild great apes (Goossens et al., 2006; Nater et al., 2011), are mainly from the telogen (quiescent) phase (Jeffery et al., 2007). Courtois et al. (1995) found that human hairs were shed two to three months after the beginning of the telogen phase. Thus, hairs shedding naturally at the same time should represent roughly the same time window. This may also apply to orangutan hairs and would thus allow segmental analysis of a bundle of shed hairs. However, nest hair originates from various, unknown body regions. It is therefore important to confirm that the incorporation of cortisol into hair is constant within and across body regions and that HCC is not influenced by local blood circulation or other unknown body region-specific factors.

In order to evaluate the utility of hair cortisol analysis for the use in captive and wild-living orangutans (*Pongo spp.*), the current study set out to provide a careful examination of several fundamental prerequisites. Specifically, we examined (I) the influence of sex and age on mean HCC, (II) the stability of cortisol concentrations in

orangutan hair over the whole hair shaft to control for systematic cortisol leaching over time and external contamination, and (III) whether body region had a significant influence on HCC in orangutans. To assess the feasibility of using segmental hair analysis on an individual level, we furthermore investigated (IV) the hair growth rate in orangutans to enable assignment of specific hair segments to their corresponding time window. Finally, to validate hair cortisol analysis in orangutans, we examined whether (V) highly stressful periods were retrospectively reflected in corresponding hair segments over a prolonged period of time on an individual case level.

2.3 Methods

2.3.1 Animals

Samples were collected from a total number of 71 captive orangutans (38 males, 33 females) from 26 European zoos (males: mean age = 22 years, range = 1 - 54 years; females: mean age = 24, range 4 - 52 years). Keepers and curators filled out questionnaires for all individuals, including information on age, sex, group composition, ranking, weight (if possible) and periods that were assumed to be stressful for animals during the last two years. The latter periods included major changes in group composition with intra-group conflicts, transfer and severe/chronic illness. Based on these subjective keeper reports, animals with perceived stressful periods were defined as 'stressed' animals (*n = 15)* whereas those without perceived stressful periods were defined as 'non-stressed' animals (*n* = 56).

2.3.2 Sample collection and preparation

Hair growth rate was assessed from three animals aged 29 (male), 26 (female) and 1 (male) by shaving and re-shaving of the same patch 4-6 weeks later. Growth rate was estimated as the regrown hair length divided by the number of days following shaving. For practical reasons, hair growth rates were obtained from different body regions.

For 62 animals, hair samples were cut approximately 1 cm above the skin (up to 8 samples from different body regions per individual). For 9 additional animals, hairs were collected from sleeping sites, resulting in a total number of 71 animals. To test for the general stability of HCC along the hair shaft, hair samples of 18 non-stressed animals (random body regions) with at least 15 cm long hair were cut into segments of 3 cm prior to analysis.

Furthermore, all hair samples with a sufficient amount of material and at least 9 cm length were cut into segments of 3 cm in order to examine whether time-limited stressors resulted in higher variation of HCC across segments (animals with time-limited stressors: $n = 10$; animals with stable living conditions: $n = 29$).

In addition, samples of three individuals met the criteria for segmental hair analysis with temporal assignment. These individuals provided hair of at least 9 cm length and furthermore had experienced severe stressful periods of at least one month. Samples including at least 100 single hairs per strand were segmented into 2 or 3 cm. Subsequently, each segment was treated as described above and HCC was plotted against the individual corresponding timeline.

To examine potential differences in HCC between different body regions we examined hair samples from six defined regions. For practical reasons, we included all animals which provided samples from three ($n = 5$), four ($n = 1$), five ($n = 6$) and six ($n = 5$) of the defined body regions. This resulted in a total number of 78 samples from 17 animals in this part of the study (right wrist upside: $n = 12$, left wrist upside: $n = 12$, stomach: $n = 14$, central back: $n = 11$, right shoulder: $n = 15$, left shoulder: $n = 15$). HCC of each hair strand was measured over the full length of hair. However, when different hair strands of the same animal showed considerable variation in length, longer strands of hairs were adjusted in length to match the shortest strands. Therefore, the examined time window could differ between individuals but was homogeneous within individuals. Hair cortisol analysis

For hair cortisol analysis, a slightly modified protocol from Stalder et al. (2012b, part study II) was followed. Samples were washed twice with 3 ml of isopropanol and dried over night. For hormone extraction, a strand of at least 100 hairs was minced into 3-5 mm pieces in order to increase the stability of results (Fourie, 2012). 10 mg of this pool were incubated with 1.8 ml of methanol for 18 hours at 45 °C. Subsequently, 1 ml of the extract was dried and resuspended in 400 µl phosphate buffer. Cortisol concentrations were determined using a commercially available immunoassay with chemiluminescence detection (CLIA, IBL-Hamburg, Germany). Intra- and inter-assay coefficients of variation of this assay are below 8%.

2.3.3 Statistical analysis

Hair cortisol data were not found to be normally distributed. Logarithmic transformations most effectively reduced the skewing of distributions and were applied for inferential analyses. For descriptive purposes, information on mean

values and standard deviations are presented in original units (pg/mg). Three animals died without signs of sickness at old age (> 49 years) within ten months after hair sampling. All of them were males and exhibited markedly increased hair cortisol values of at least two standard deviations above the mean of non-stressed individuals. As the underlying long-term endocrine mechanisms during that stage of life are largely unknown, these individuals were excluded from subsequent analyses in order to avoid false positives. For the between-subject examination on general effects of sex, age and perceived long-term stress (> 1 month) on HCC, individual hair cortisol levels were calculated as a mean of all available hair samples for each animal. A Pearson correlation was run to examine the relationship between age and HCC. To identify effects of sex and perceived stress on HCC, a 2 x 2 analysis of variance (ANOVA; male vs. female and stressed vs. non-stressed) was conducted. Because juveniles still enjoy a high degree of freedom in their behavior the respective effects were mainly expected in adults. Therefore, juveniles (< 10 years, Weingrill et al., 2011) were excluded from this particular analysis.

A repeated-measures ANOVA was used to test for differences in HCC across hair segments. As this analysis aimed to study the stability of HCC under stable living conditions, only hair samples of animals were included that had no perceived major stressful periods over the examined period.

To investigate whether unstable living conditions with time-limited stressors (1-3 months) resulted in a generally increased HCC variation between segments, a two-tailed Student *t*-test was conducted comparing animals living in stable conditions with those animals with time-limited stressors. In this analysis, the coefficient of variation (CV%) across different hair segments was used as dependent variable.

The comparison of HCC between different body regions was conducted using a repeated-measure ANOVA with Greenhouse-Geisser corrections being applied to account for violation of sphericity. Because repeated-measures ANOVA would discard incomplete sets of data, we increased the statistical power for this analysis by replacing missing HCC values of individual body regions (n = 23 of 102) by use of a multiple imputation as recommended by others (Rubin, 2009, Schafer and Graham, 2002). All statistical analyses were conducted with SPSS for windows, version 19 (IBM, Chicago, IL).

2.4 Results

2.4.1 Influence of sex, age and stress on HCC

The mean ± SD HCC values of all examined individuals were 28 ± 18.6 pg/mg (range: 9 to 108 pg/mg; males: 28 ± 18.6; females: 20 ± 6.4 pg/mg; juveniles: 19 ± 7.6 pg/mg). The mean ± SD HCC of the males that died of old age were 70.3 ± 32.7 pg/mg (range: 40 to 105 pg/mg) and those data were excluded from subsequent analyses. HCC was found to be unrelated to age (r = 0.12, P = 0.34, n = 68). The two-way (stress x sex) ANOVA revealed a main effect of stress ($F_{(1,\ 64)}$ = 23.92, $P < 0.001$, η^2_p = 0.27; Figure 2.1), illustrating significantly higher HCC in stressed animals (43.6 ± 26.5 pg/mg, n = 13) than in non-stressed animals (19.3 ± 5.5 pg/mg, n = 55). No main effect of sex ($F_{(1,64)}$ = 3.7, P = 0.06, η^2_p = 0.06) or a stress x sex interaction ($F_{(1,64)}$ = 0.4, P = 0.52, η^2_p = 0.01) were found.

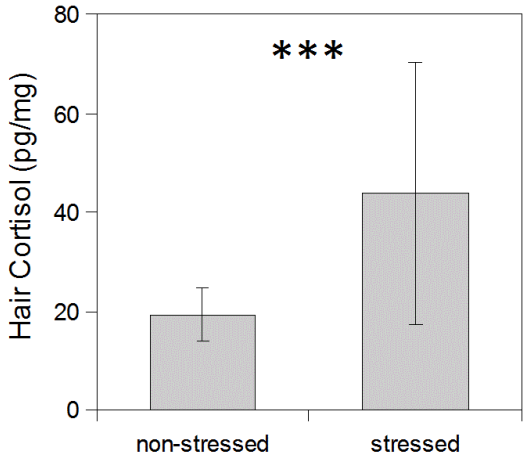

Figure 2.1: Mean hair cortisol concentration (with standard deviation) of orang-utans without perceived stressful periods ('non-stressed': n = 55) and with perceived stressful periods ('stressed': n = 13).

2.4.2 Cortisol stability and variability along hair shaft

In non-stressed animals with hair strands of 15 cm, no differences in HCC were found between the five consecutive 3 cm hair segments ($F_{(2.6,\ 44.7)}$ = 1.6, P = 0.2, n = 18; Figure 2.2). In line with this, the examination of HCC in a single animal with very long hair (42 cm representing ~3.5 years) also revealed a very stable HCC profile across all 14 segments (35.2 ± 2.5 mg/pg). Furthermore, the HCC between segments varied significantly more in hair samples of animals for which keepers had reported some stressful periods (CV% = 32.8 ± 16.5%, n = 10) than in animals which had lived under stable conditions (CV% = 16.1 ± 9%, n = 29; $t_{(37)}$ = 3.79, $P < 0.001$).

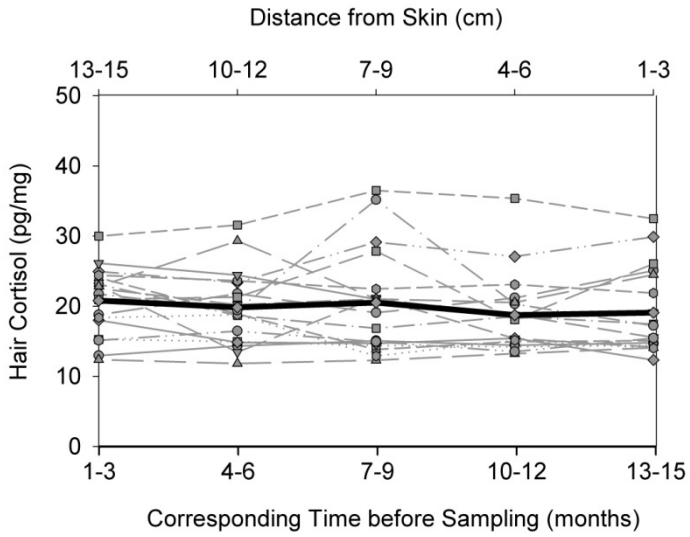

Figure 2.2: Hair cortisol profiles of individual orang-utans with a hair length ≥ 15 cm (n = 18). HCC are shown for five consecutive segments of 3 cm. Each data point represents a mean HCC of about 3 months. Mean values of all 18 profiles are illustrated by a solid black line.

2.4.3 Influence of body regions on HCC

HCC did not differ between the six defined body regions ($F_{(2.4,\ 38.1)}$ = 1.10, P = 0.4, n = 17 animals). Figure 2.3 shows for each of the six defined body regions the mean percentage deviation from the animal's mean HCC, where in each comparison the mean HCC was based on all other body regions except the one being compared (right wrist: -6 %; left wrist: -7 %; stomach: 0 %; back: 1 %; right shoulder: 6 %; left shoulder: 7 %). For all 28 animals of which hair samples from two or more body regions were available, the mean CV% of HCC between body regions was 14.3% (range = 4.7% to 29.6%).

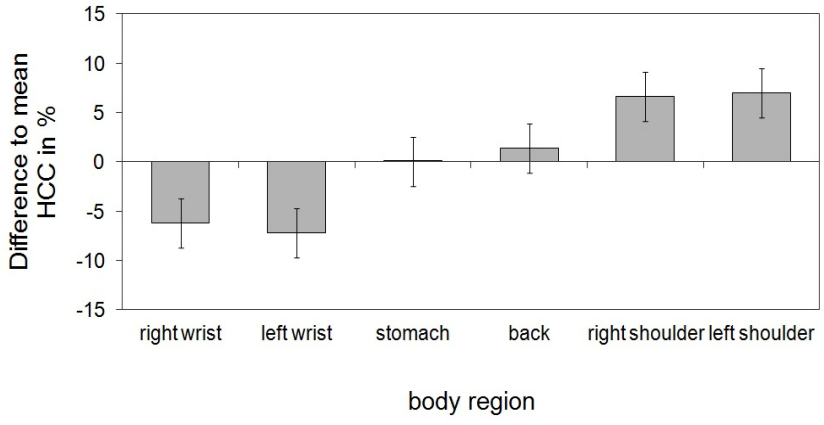

Figure 2.3: Percentage difference (with standard error) between defined body regions and the mean hair cortisol concentration (HCC, excluding the respective body region). There was no significant difference between body regions. Values are based on a total of 79 samples from 17 orang-utans: n (right wrist) = 12, n (left wrist) = 12, n (stomach) = 14, n (back) = 11, n (right shoulder) = 15, n (left shoulder) = 15.

2.4.4 Hair as a retrospective endocrine calendar – individual case reports

The mean growth rate was found to be 0.98 cm / 30 days (individual 1: 1.1 ± 0.13 cm / 30 days; individual 2: 0.89 ± 0.18 cm / 30 days; individual 3: 0.95 ± 0.16 cm / 30 days; Figure 2.4).

Based on the assessed hair growth rate, Figure 2.4 (Individual A) shows the hair cortisol profile of a 44-year old female that was frequently attacked by another female group member. Physical aggression toward A increased and culminated in 2009. The keepers therefore repeatedly changed group composition in order to handle the situation. Plotting the hair cortisol profile against the time axis revealed 4-5 fold elevated HCC compared to non-stressed animals (86 pg/mg) in times of exposure to direct aggression in February-April 2010 (i.e. serious biting, chasing, food stealing) and 2-3 times elevated cortisol levels (53 pg/mg) in times of exposure to indirect or psychological aggression in August-October 2010 (permanent contact to main aggressor through iron bars, behavioral displays of the main aggressor). On the other hand, HCC dropped to average levels in a period of total isolation from other orangutans or during reintegration to the group after removal of the main aggressor.

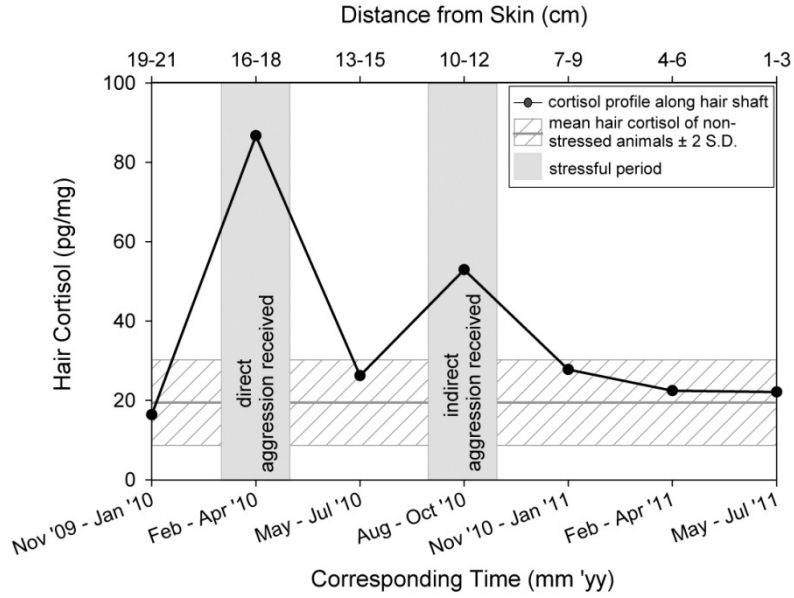

Figure 2.4: Cortisol profile along one hair strand of individual A. Each data point represents the mean cortisol concentration of a three months period. Estimated individual hair growth rate = 1.0 cm / month.

Figure 2.5 illustrates the HCC profile of individual B, a 29-year-old female orangutan that, by coincidence, used to be the main aggressor of A. The cortisol profile of B retrospectively revealed that HCC was elevated 3-4 fold above the average level of non-stressed animals (75 pg/mg) during times of total isolation (May-June 2010) and an operation (removal of uterus: July 2010). However HCC remained

at a high level when B was reintegrated into the group with indirect contact through iron sliders with A. HCC levels even increased up to 5 fold over the average values (103 pg/mg) after the transfer to another zoo in October 2010, but finally decreased over the following nine months when the animal increasingly habituated to the new environment.

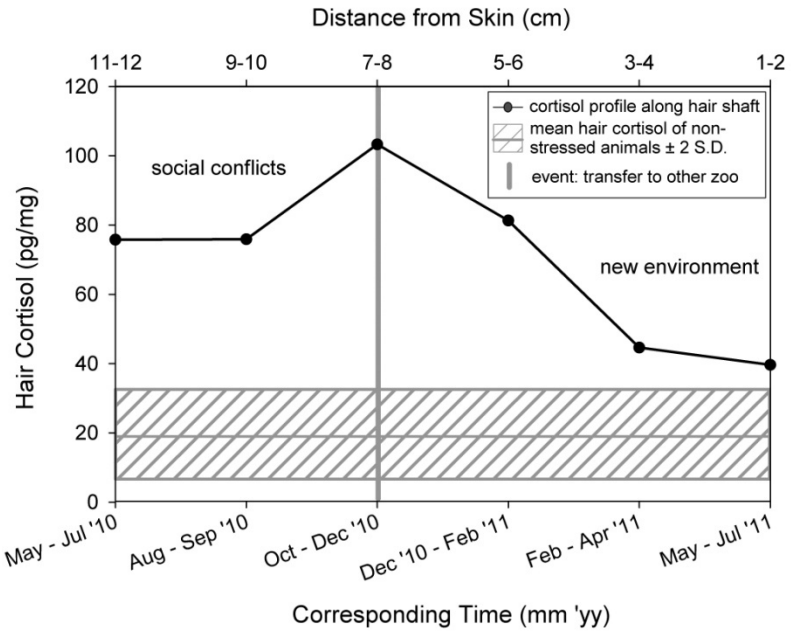

Figure 2.5: Cortisol profile along one hair strand of individual B. Each data point represents the mean cortisol concentration of a two months period. Estimated individual hair growth rate = 0.9 cm / month.

Figure 2.6 shows the hair cortisol profile of a one-year-old baby orangutan. His mother died of chronic airsacculitis when he was eight months old (February 2011). The orphan was adopted by another female of the group about two weeks after the mother's death (March 2011). After the adoption, HCC reduced from 3-fold the average values (64 pg/mg) to levels of non-stressed animals (28 pg/mg).

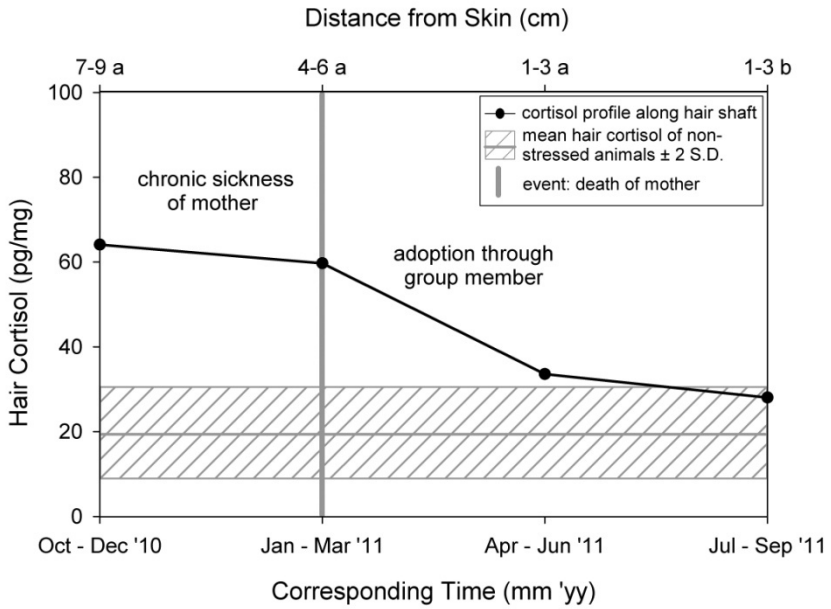

Figure 2.6: Cortisol profile of two strands of hairs of a one-year old orphaned baby orang-utan (hair strand b was cut three months after a). Each data point represents the mean cortisol concentration of a three months period. Measured individual hair growth rate = 0.95 cm/month.

2.5 Discussion

The current study on orangutans shows that the analysis of the stress-responsive hormone cortisol in hair is a highly valuable tool, which may advance long-term stress monitoring. Specifically, the current results support the notion that a retrospective calendar of cumulative cortisol secretion may be derived from segmental hair analyses across the whole length of hair. This was supported by the finding that there is no HCC decrease over time in captive orangutans and that HCC in proximal to distal segments remains stable even when hair reached lengths of more than 40 cm. This is in accordance with previous studies on HCC in animals (Bennett and Hayssen, 2010; Davenport et al., 2006; Macbeth et al., 2010) but contrasts with the waning effect that was found towards distal hair segments in some research on human hair (D'Anna-Hernandez et al., 2011; Gao et al., 2010; Kirschbaum et al., 2009; Steudte et al., 2011). Further studies may be warranted to investigate the effects of frequent rain on the concentration of hair cortisol in free-ranging orangutans as indicated by first results of *in vitro* research on pooled hair samples from rhesus macaques (Hamel et al., 2011) and humans (Li et al., 2012).

Equally important were our findings of discrete HCC peaks in hair segments corresponding to the time of perceived stress. Previously, only a small number of studies on human scalp hair suggested that segmental hair analyses reflected at least 1.5 years of the clinical course of patients treated for pathological hyper- or

hypocortisolism (Manenschijn et al., 2012, 2011; Thomson et al., 2010). The only other attempt to provide a retrospective cortisol calendar from segmental hair analysis in animals was made in horses. However, this trial failed, which was potentially due to external contamination through sweat (Anielski, 2008). The cortisol profiles of our three orangutan case studies are therefore the first that show the feasibility of segmental hair analysis in animals. This reflects several key points. First, cortisol molecules are mainly incorporated into the matrix of orangutan hair during or shortly after the formation and second, the molecules do not move along the hair shaft after the incorporation, unlike cocaine molecules (Henderson, 1993). Third, the clear cortisol peaks in distant segments indicate that neighboring hairs grow at the same rate, and therefore, reflect the same time window. Finally, the present study shows that in captive orangutans the length of the retrospective cortisol calendar is only dependent on the length of hair, with longer hairs reflecting longer time windows.

The orangutans have long hair, but working with short hair requires particular attention. Samples of shorter hair will contain a smaller percentage of hair in the anagen (growth) phase and an increased percentage of telogen, non-growing hair (Courtois et al., 1995). Because anagen and telogen hair vary in their corresponding time window, one segment consisting of multiple anagen and telogen hairs, integrates different time windows within a single segment. Therefore, segments of neighboring hairs are unlikely to represent the same time window in short haired animals or body regions. In order to still enable the application of segmental hair analysis to shorter hair, we suggest that the longest (telogen) hair could be removed prior to the analysis.

This is the first study to show the feasibility of segmental hair analysis using non-human primate hair. One strand of approximately 100 hairs enabled us to track the cortisol level of individuals for up to several years, given an adequate length of hair. The observed results highlight the potential of this method for the application in animal welfare to objectively judge keeping conditions, group composition and translocation success. However, in order to fully apply segmental hair analysis, more research on hair growth is necessary. To our knowledge, the present study provides the first data on hair growth rates in orangutans. There is limited information on hair growth rates in other primate species (Fourie, 2012). Our results indicate that hair growth rates in all three tested orangutans were similar to that in humans. In contrast, Fourie (2012) reported that rates in two gorillas (*Gorilla gorilla gorilla)* varied from

1.2 cm/month to 5.8 cm/month. However, we belief this degree of variation on growth rates in the same species is unlikely and is potentially due to methodological issues.

The baseline cortisol levels indicated that sex did not appear to have significant influence on HCC. This is in line with results from fecal glucocorticoid in orangutans (Weingrill et al., 2011). Furthermore, our results did not show any age-related HCC pattern. Very few other studies investigated the effect of age on the baseline cortisol levels in non-human primates. Weingrill et al. (2011) found slightly increased faucal glucocorticoid concentrations in older individuals. Similar to our findings, Sapolsky and Altmann (1991) generally found no influence of age on cortisol concentration in yellow baboons but described hypercortisolism in the oldest individuals (> 16 years). In contrast, Fourie and Bernstein (2011) described infant hypercortisolism in vervet monkeys and guinea baboons. However, due to missing samples of small infant orangutans (<1 year) our data does not allow any conclusion on this matter.

Our results suggest that body region did not have a significant influence on HCC in orangutans. This is an important precondition for the application of hair cortisol measurement in wild orangutans which requires the use of naturally shed hair found in sleeping nests (Goossens et al., 2006; Jeffery et al., 2007; Nater et al., 2011). Besides representing a different time window than cut (anagen) hair, shed hair is likely to originate from different body regions. Our results on the influence of body region on HCC are in accordance with results found in rabbits (Comin et al., 2012) but contrast with one animal study that showed significantly higher HCC in the neck region in grizzly bears (Macbeth et al., 2010). However, further studies are required to find out if this reflects a species-specific process. Most importantly for the application of hair cortisol measurement in wild orangutans, the coefficient of variation for different samples of the same animal was comparatively low. This will allow the use of shed hair which is a mix of different body regions.

In conclusion, this study shows that orangutan hair can be used as a long-term retrospective cortisol calendar for stress monitoring. Therefore, segmental hair cortisol analysis is a powerful tool for captive management. Furthermore, our results encourage the use of naturally shed hair to assess basal cortisol concentrations. Because shed hair in nests can be obtained non-invasively and even from animals which are not habituated this will open new possibilities in field endocrinology and conservation.

2.6 Acknowledgements

This study could be successfully conducted only with the enduring assistance of animal keepers, veterinarians and curators from the zoos in Frankfurt, Madrid, Qiryat Motzkin and Zürich, but also those in Aalborg, Amneville, Apeldoorn, Boissière, Borås, Chester, Colchester, Dresden, Duisburg, Furuvik, Gelsenkirchen, Gran Canaria, Heidelberg, Krefeld, Les Mathes, Liberec, Nantes, Neunkirch, Paris, Rostock, Santillana, Romanèche-Thorins and Usti nad Labem. We would also like to thank Clemens Becker, the orangutan studbook keeper of the EEP, for facilitating contact with these zoos. Finally, we would like to thank Antje Tietze and the Dresden HairLab Team and Tony Weingrill for support, discussions and comments throughout this study. Funding for this study was kindly provided by the Jane Goodall Institute Switzerland to Esther Carlitz.

3. Effects of body region and time on hair cortisol concentrations in chimpanzees (*Pan troglodytes*)

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3.1 Abstract

Hair cortisol concentrations (HCC) are increasingly recognized as an integrated measure of the systemic cortisol secretion. Yet, we still know very little about confounding effects on HCC in animals. The present study therefore used hair from semi-wild and zoo living chimpanzees to investigate (1) intra-individual variability of HCC (body-region effect), and (2) the stability of HCC along the hair shaft (traditionally called the washout effect). Our results indicate that absolute HCC varied substantially between certain body regions, but a factor analysis revealed that these HCC differences were mainly attributable to one common source of variance. Thus, hair from all body regions provides similar biological signals and can be mixed, albeit at the cost of a lower signal-to-noise ratio. With regard to potential underlying mechanisms, we studied skin blood flow, as observed through thermal images from one chimpanzee. We found the general HCC pattern was reflected in differences in surface body temperature observed in this individual in three out of four body regions. In a separate set of samples, we found first evidence to suggest that the systematic cortisol decrease along the hair shaft, as observed in humans, is also present in chimpanzee hair. The effect was more pronounced in semi-wild than in zoo chimpanzees presumably due to more exposure to ambient weather conditions.

3.2 Introduction

Cortisol concentration measured in hair is increasingly recognized as a measure of long-term stress. Recently, the repetitive ACTH application in cows, chipmunks and lynx resulted in significantly increased HCC has provided direct evidence that hair cortisol concentrations (HCC) reflect the integrated activity of the hypothalamic-pituitary-adrenal axis (del Rosario Gonzalez-de-la-Vara et al., 2011; Mastromonaco et al., 2014; Terwissen et al., 2013). Hair cortisol analysis is increasingly applied in various circumstances, including clinical diagnosis in livestock (Comin et al., 2013), behavioral science (Dettmer et al., 2014), captive management (e.g., Carlitz et al., 2014), and conservation (e.g., Bryan et al., 2013). Hair cortisol analysis has also been applied in a growing number of mammalian species, including humans (review: Staufenbiel et al., 2013), non-human primates (e.g., Carlitz et al., 2014; Davenport et al., 2008, 2006; Dettmer et al., 2014; Fourie and Bernstein, 2011; Yamanashi et al., 2013), bears (e.g., Bechshøft et al., 2012, 2011; Macbeth et al., 2012, 2010; Malcolm et al., 2013), cows (Moya et al., 2013), horses (Anielski, 2008), dogs (Accorsi et al., 2008; Bennett and Hayssen, 2010), rock hyraxes (Koren et al., 2008), lynx (Terwissen et al., 2013) and chipmunks (Martin and Réale, 2008; Mastromonaco et al., 2014).

Despite the increased application of HCC analysis, the indicator value of HCC would be greatly improved if we knew which other factors than long-term stress affect the incorporation of and retention of cortisol in hair. Unfortunately, we insufficiently understand the factors that may confound HCC values. Various factors have been suggested, such as a systematic decline along the hair shaft (the so-called washout effect), systematic variation among body regions (body-region effect), or external contamination through sweat.

The present study attempts to fill those methodological gaps of HCC assessment in chimpanzees. While the influence of sweat is debated for humans (Grass et al., 2015; Russell et al., 2013) and horses (Anielski, 2008), it is rather unlikely to affect great ape species since their active sweat glands are mainly restricted to their palms, forehead and auxiliary organs (Montagna, 1972). We therefore examined in chimpanzees whether HCC show systematic variation across different body regions and whether HCC decreases along the hair shaft.

The washout effect has been observed in several human studies (Dettenborn et al., 2010; Gao et al., 2010; Kirschbaum et al., 2009; Skoluda et al., 2012; Steudte et

al., 2011, but see Manenschijn et al., 2011; Thomson et al., 2010). It was initially suggested that structural damage of distal hair segments might permit liquids like water and cosmetics (Kirschbaum et al., 2009), or alcohol used to clean the samples before analysis (Manenschijn et al., 2011), to penetrate and wash out cortisol more easily. This would be reflected in decreasing HCC from skin-near to distal parts of the hair. While evidence for this assumption from *in vivo* studies is still pending, *in vitro* experiments by Li and colleagues (2012) showed that hair treated with shampoo solution, warm or hot water only, as well as UV-irradiation resulted in significant HCC loss, suggesting that cortisol may not only leach from the hair shaft but that it may also be degraded through UV-irradiation as was shown earlier for cannabinoids in hair (Skopp et al., 2000). Therefore, the term “waning effect” may be more appropriate than the traditionally used term “washout effect”, which was meant to indicate the leaching of cortisol by water (Kirschbaum et al., 2009).

Concerning the waning effect in animal hair, an HCC decrease by water was shown only in one *in vitro* experiment where human-like extensive wash-dry procedures resulted in decreased HCC in hair from rhesus macaques (Hamel et al., 2011). All *in vivo* animal studies showed no HCC difference between the proximal and distal end of hair (Bennett and Hayssen, 2010; Carlitz et al., 2014; Davenport et al., 2006; Yamanashi et al., 2013). The latter findings may be in line with the *in vitro* experiments on hair of humans and rhesus macaques because the captive subjects were not exposed to rain and presumably not to strong UV-irradiation either. A systematic HCC decrease along the hair shaft should only be expected if hair is frequently exposed to water (Hamel et al., 2011), or UV-light (Li et al., 2012). However, Macbeth et al. (2010) also found no systematic decline along the hair shafts of water and light exposed free ranging grizzly bears, which raises the question whether some animal hair is inert to systematic HCC decrease along the hair shaft. The present study therefore investigated whether systematic cortisol decrease existed in chimpanzee hair and, if present, whether the effect could be explained by exposure to water only.

Concerning the body-region effect, an increasing number of studies suggest that HCC differs among various body regions in animals (Macbeth et al., 2010; Moya et al., 2013; Terwissen et al., 2013; Yamanashi et al., 2013, but see Carlitz et al., 2014; Comin et al., 2012; Macbeth et al., 2012), or between head regions in humans (Li et al., 2012). However, to our knowledge it has never been investigated whether HCC

from all body regions provide similar biological information and what underlying mechanism may lead to this body-region effect. Yet, a better causal understanding of the effect may improve our understanding of the cortisol incorporation mechanisms into hair, which is still poorly understood (review: Meyer and Novak, 2012; Russell et al., 2012; Stalder and Kirschbaum, 2012).

Following the multiple compartment model of mechanisms of drug incorporation into hair (Henderson, 1993), cortisol in hair is discussed of being enriched from sweat cortisol (Russell et al., 2013, but see Grass et al., 2015), from sebum, or from local cortisol production in the skin (Pang et al., 2014; Rousseau et al., 2007; Slominski et al., 2013, 2007) and hair follicles (Ito et al., 2005). Another mechanism suggested to contribute to the incorporation of cortisol into hair is the passive diffusion from the supplying blood capillaries into the growing hair cells (reviewed in: Meyer and Novak, 2012; Russell et al., 2012; Stalder and Kirschbaum, 2012), which would be in line with the fact that multiple weekly ACTH injections resulted in increased HCC in the hair segments representing the time of application (del Rosario Gonzalez-de-la-Vara et al., 2011; Mastromonaco et al., 2014; Terwissen et al., 2013). Following this idea, a higher activity of the hypothalamic-pituitary-adrenal axis leads to higher cortisol concentrations in the blood stream, which results in higher cortisol incorporation into the hair shaft due to increased diffusion pressure. In parallel, it is conceivable that differences in skin blood flow (SkBF) can influence the cortisol uptake into hair in a similar process. Increased SkBF may increase cortisol availability per time unit, which could result in higher diffusion rates and thus higher cortisol incorporation into the growing hair. Several studies on humans and various animal species have documented differences in SkBF between body regions using laser Doppler velocimetry or photopulse plethysmography (Monteiro-Riviere et al., 1990; Tsuchida, 1987; Tur et al., 1983). Because higher SkBF leads to increased heat dissipation, differences in SkBF can be indirectly measured as skin temperature (Rubinstein and Sessler, 1990). In order to test this skin blood flow hypothesis, the present study compared the HCC in four different body regions with their skin temperatures in one female chimpanzee.

We obtained hair cortisol measures from European zoo chimpanzees as well as from semi-wild living sanctuary chimpanzees (Ngamba Island, Uganda) from various body regions in two consecutive years. Longitudinal segmentation of hair samples allowed investigating HCC measures along the hair shaft. Because the semi-wild

chimpanzees were far more exposed to sun and rain than the zoo-living conspecifics, we could also examine whether this difference affected the waning effect.

3.3 Methods

3.3.1 Hair sampling and animals

Hair samples were cut from semi-wild living chimpanzees from the Ngamba Island (NI) sanctuary (Uganda) during anesthesia for the annual routine health checks in late February to early March 2011 (18 males aged 8-28 years, 20 females aged 5 to 28 years) and in late March 2012 (1 male aged 20, 9 females aged 9 to 25 years). These animals live in a 40 ha forest during the day and are encouraged with food to sleep in the roofed holding facilities at night. Hair samples were cut from six different body regions per individual (right and left dorsal forearm, center of back, right and left shoulder blade, middle of chest; there were 9 missing samples: right or left shoulder blade in 5 animals, right forearm in 1 animal, back in 1 animal, back and chest in 1 animal). For investigations on HCC stability along the hair shaft, additional hair samples were collected from 78 chimpanzees from 19 European zoos. However, only 24 samples from 13 zoos provided sufficient length and amount of material to be included in the segmentation study. Those samples were cut from the shoulder region (3 males aged 16 and 33 years, 6 females aged 3 to 43 years), back (3 males aged 30 and 41 years, 3 females aged 14 to 17 years), forearm (5 males aged 10 and 43 years) or combed from across several regions (3 males aged 17 and 36 years, 1 females aged 17 years) either in cooperation with well-trained animals (n = 16) or during anesthesia (n = 5) due to medical treatment.

3.3.2 Hair cortisol analysis

For studies on body-region effect, the 3 cm of hair proximal to the skin were analyzed, whereas for investigations on HCC stability along the hair shaft, the 4 cm of hair proximal to the skin were cut into 1-cm-segments. Here, all hair strands that provided sufficient length and amount of material were included in the analysis (n_{Zoo} = 24, n_{NI2011} = 46, n_{NI2012} = 25).

For cortisol analysis, all subsamples were washed twice for three minutes with 3 ml isopropanol. The air-dried hair was then minced into 3-5 mm pieces and 6 mg of this 10-50 mg pool were incubated with 1.8 ml methanol for 17 h in a glass tube. 1.6 ml of the extract were transferred into another glass tube, dried and re-

suspended in 150 µl Aqua bidest. Cortisol concentrations were determined using a commercially available immunoassay with luminescence detection (LIA, IBL-Hamburg, Germany). Intra- and inter-assay coefficients of variation of this assay are below 8%.

3.3.3 Heat dissipation measures

Thermal images from the front and the back were taken from one female zoo chimpanzee using a thermographic camera (Vario Cam® high resolution, InfraTec GmbH, Dresden, Germany). The Software IRBIS ® 3 (InfraTec) was employed to extract temperature measures from the chest, forearm, shoulder blade and back according to the hair sampling points from the Ngamba Island chimpanzees. Measure points were directed to areas with little hair, which exhibited the highest temperature in order to avoid false measures due to the insulating effect of fur. For each body region, the mean temperature with standard deviation was derived from a small circular area (Figure 3.5).

For this study, it was not possible to add more thermal images from other animals because chimpanzees had either too much fur which prevented us from obtaining clear measures or there was no thermographic camera with high resolution available in the facilities.

3.3.4 Statistical analysis

Hair cortisol data was not found to be normally distributed. Fourth root transformation reduced the skewness of distribution most effectively and was applied prior to data analyses (Miller and Plessow, 2013). For descriptive purposes, information on mean values and standard deviations are presented in original units (pg/mg). All analyses were performed using R 3.1.1 (R Core Team, 2015) statistical software.

3.3.4.1 HCC stability along hair shaft

Mixed-effects linear regression models were employed to assess potential HCC changes across the four consecutive segments in both zoo and NI samples, while accounting for systematic variance due to different chimpanzees and body regions. Thus, the stability of HCC across the four consecutive segments could be quantified by means of intra-class correlation coefficients (ICC; see Hruschka et al., 2005). The upper bound of such ICCs (i.e. ICC = 1) indicates perfect stability of HCC across

segments (i.e., the rank order of HCCs from the first segments is also preserved across the subsequent segments), whereas the lower bound (ICC = 0) indicates no association between the different segments. The employed model is expressed in equation 1:

$$HCC^{0.25}_{ijk} = \beta_0 + \beta_1 \times Segment + \xi_i + \zeta_{ij} + \varepsilon_{ijk} \quad (1)$$

where $ß_0$ denotes $HCC^{0.25}$ in the first segment, $ß_1$ denotes the change of $HCC^{0.25}$ per subsequent segment. The random effects ξ_i and ζ_{ij} account for the variation in $HCC^{0.25}$ due to differences between individuals *i* and body-regions *j*, respectively.

3.3.4.2 HCC in different body regions

For descriptive purposes, Pearson correlation coefficients were calculated to investigate the HCC relationships between the different body regions. Thereafter, a confirmatory factor analysis was performed (Rosseel, 2012) to investigate whether the covariance structure of HCC across different body regions could be sufficiently accounted for by one common factor. Besides the robust likelihood ratio statistic (Bentler and Yuan, 1999), model fit was evaluated using the comparative fit index (CFI), root mean square error of approximation (RMSEA), standardized root mean square residual (SRMR) and the Bayesian information criterion (BIC). HCC differences between the investigated body regions were assessed using paired *t*-Tests.

3.4 Results

3.4.1 HCC stability along the hair shaft

Pair-wise comparison revealed that all four segments were highly correlated in both samples from the zoos and from Ngamba Island ($0.9 \geq r \geq 0.7$, all $p's < 0.001$). Mixed-effects regressions of HCC on the four consecutive 1-cm-segments revealed profound differences between change of HCC in chimpanzee hair from European zoos and those from the NI sanctuary. In zoo chimpanzees, we found a decrease of HCC across all segments that reached statistical significance ($\chi^2(1) = 4.22$, $p = 0.040$) but was numerically negligible (mean ± SD decrease of HCC per segment: 5.7% ± 18.8%; 61.1% explained variance). The intra-individual stability of HCC across the four segments amounted to ICC = 0.81. Hair samples from NI-chimpanzees showed a more pronounced cortisol decline along the hair strand ($\chi^2(1) = 72.40$, $p < 0.001$; mean ± SD decrease of HCC per segment: 20.1% ± 24.9%;

69.6% explained variance). However, the intra-individual stability of HCC across the four segments was similar to that of the zoo samples, and amounted to ICC = 0.83. After controlling for body-region-related differences in HCC, the intra-individual stability estimate of HCC drops to ICC = 0.32. This is the proportion of HCC that can actually be attributed to environmental and individual stability across the time interval covered by the hair stands from different body regions of a single chimpanzee. The mean trajectories of the HCC decrease across segments, as traditionally reported in previous studies (Carlitz et al., 2014; Kirschbaum et al., 2009; Manenschijn et al., 2011), are visualized in Figure 3.1 whereas a more detailed visualization of the HCC development along segments is presented in Figure 3.2.

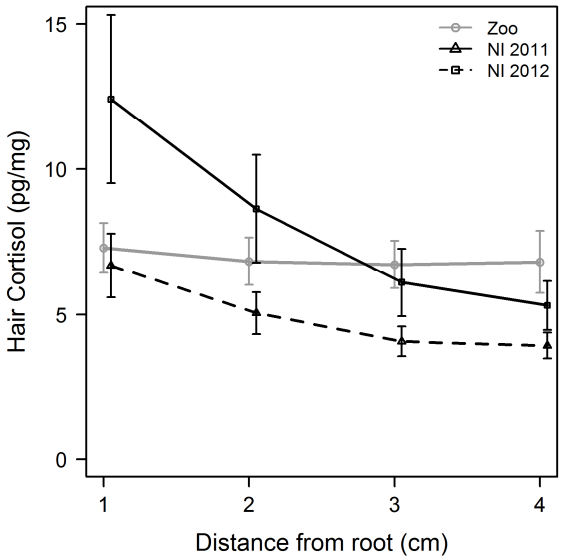

Figure 3.1: Mean hair cortisol concentrations (± SE) of the four proximal 1-cm-segments from 24 European zoos-living chimpanzees (gray line, *n* = 24 samples), 38 chimpanzees from Ngamba Island (Uganda) in 2011 (dashed black line, *n* = 46 samples), and from 7 Ngamba Island chimpanzees in 2012 (solid black line, *n* = 25 samples).

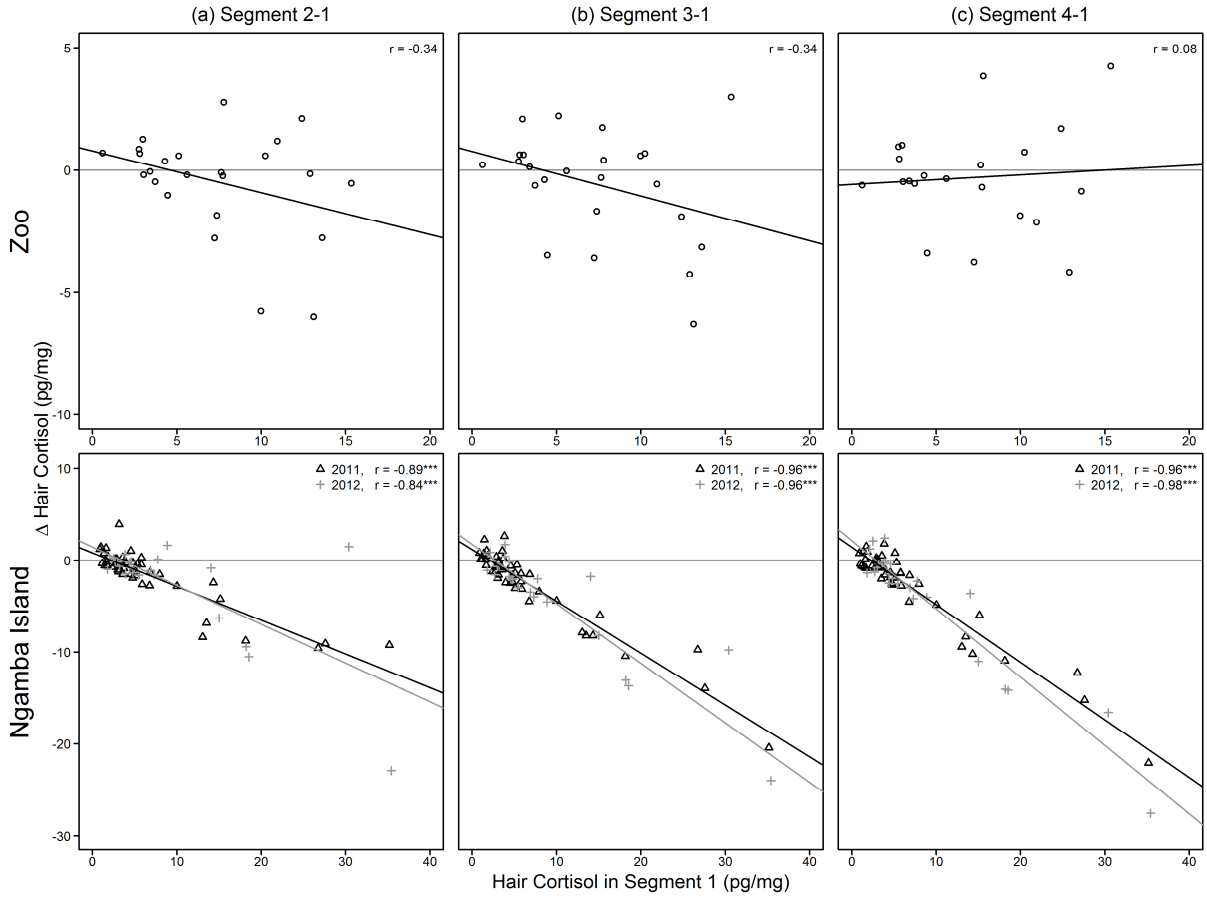

Figure 3.2: Detailed illustration of the hair cortisol changes along four consecutive 1-cm-segments for samples from European zoo chimpanzees (n = 24) and from semi-wild living chimpanzees from the Ngamba Island (NI) Chimpanzee Sanctuary, Uganda (hair samples from 2011, n = 46; hair samples from 2012, n = 25). Negative y-values indicate that cortisol concentrations (HCC) in segment 2 (a), segment 3 (b) or segment 4 (c) was smaller than cortisol concentration in segment 1. Cortisol decrease towards the distal end of hair was present in both NI ($\chi^2(1) = 72.40$, $p < 0.001$) and zoo samples ($\chi^2(1) = 4.22$, $p = 0.040$), but only NI samples exhibited significant correlations between HCC in segment 1 and the loss of HCC across all following segments. ****p < 0.001*

3.4.2 HCC in different body regions

Descriptive statistics for HCC in the different body regions are provided in Table 3.1. Pearson correlations were highly significant between all pairs of body regions (Table 3.1) and showed a strong effect size (Cohen, 1988). Results from the factor-analysis revealed that the HCC covariance structure could be sufficiently accounted for by only one factor ($\chi^2(2) = 2.07$, $p = 0.35$; CFI = 1.00, $RMSEA_{90\%}$ = 0.00 – 0.31, SRMR = 0.02, BIC = -119.74), suggesting that HCC at different body regions were driven by one systemic factor across the time interval covered by the length of hair strand. Factor loadings indicated, that chest HCC ($\lambda = 0.90$, $p < 0.01$) contributed most substantially to this "cortisol exposure" factor, followed by shoulder and forearm HCC (both λ's = 0.87, p's < 0.01), and finally back HCC ($\lambda = 0.78$, $p < 0.01$).

Constraining all loadings to equality did not result in a significantly worse model fit ($\Delta\chi^2(3) = 6.27$, $p = 0.10$ with $\chi^2(5) = 8.95$, $p = 0.11$; CFI = 0.98, $RMSEA_{90\%}$ = 0.00 – 0.25; SRMR = 0.13, BIC = -122.56). Therefore, HCC differences between animals probably are equally manifested in hair from all body regions.

Table 3.1: Pearson correlation coefficients of hair cortisol concentrations of four body regions from 48 chimpanzees. Point estimates are listed below the main diagonal of the matrix, whereas their bootstrapped 95% confidence regions are listed above the main diagonal.

	back	forearm	shoulder	chest
N	46	48	47	47
Mean	3.41	3.88	4.92	6.88
SD	1.73	2.69	3.48	5.31
Minimum	0.18	0.6	0.97	1.09
Maximum	9.28	16.82	17.01	31.49
Correlation (back)	1	[0.62, 0.82]	[0.53, 0.77]	[0.56, 0.80]
Correlation (forearm)	0.73	1	[0.57, 0.87]	[0.60, 0.88]
Correlation (shoulder)	0.67	0.75	1	[0.68, 0.89]
Correlation (chest)	0.69	0.78	0.81	1

Besides the strong correlations, the paired t-Tests revealed that the absolute HCC differed (at least marginally) significantly between all investigated regions (all *p's* ≤ 0.06; Figure 3.3). HCC was significantly higher in the chest region followed by shoulder, forearm and back. A very similar pattern was observed for temperature measures based on thermal images from one chimpanzee although only three out of four body regions followed the average HCC pattern in this particular chimpanzee (Figure 3.4 and Figure 3.5).

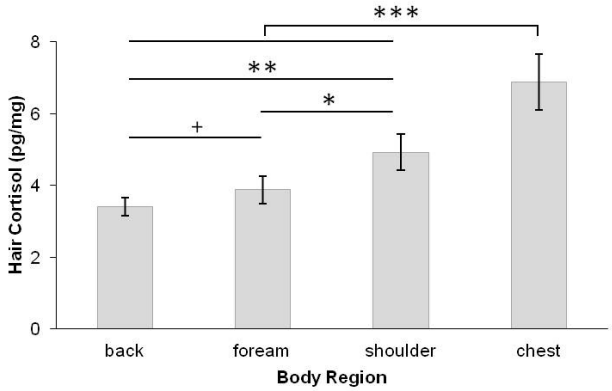

Figure 3.3: Bar plot illustrating the mean hair cortisol concentrations (±SE) of four body regions from 48 samples sets from semi-wild ranging chimpanzees from the Ngamba Island chimpanzee sanctuary (Uganda; 2011: $n = 38$, 2012: $n = 10$). Each value is an integrated measure of the three cm of hair closest to the skin. Forearm and shoulder show the mean concentrations of the right and left side. + $p = 0.06$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

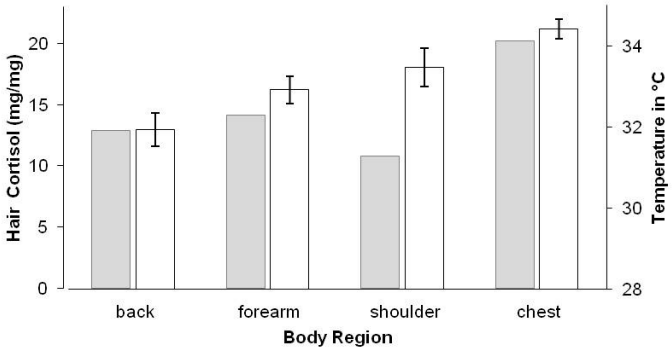

Figure 3.4: Bar plot illustrating the hair cortisol concentration (gray bars) and skin temperature (± SD, white bars) of four body regions from one female zoo chimpanzee. Each cortisol value is an integrated measure of the three cm of hair closest to the skin. Temperature measures were measured using thermal imaging. Forearm and shoulder samples were taken from the right side.

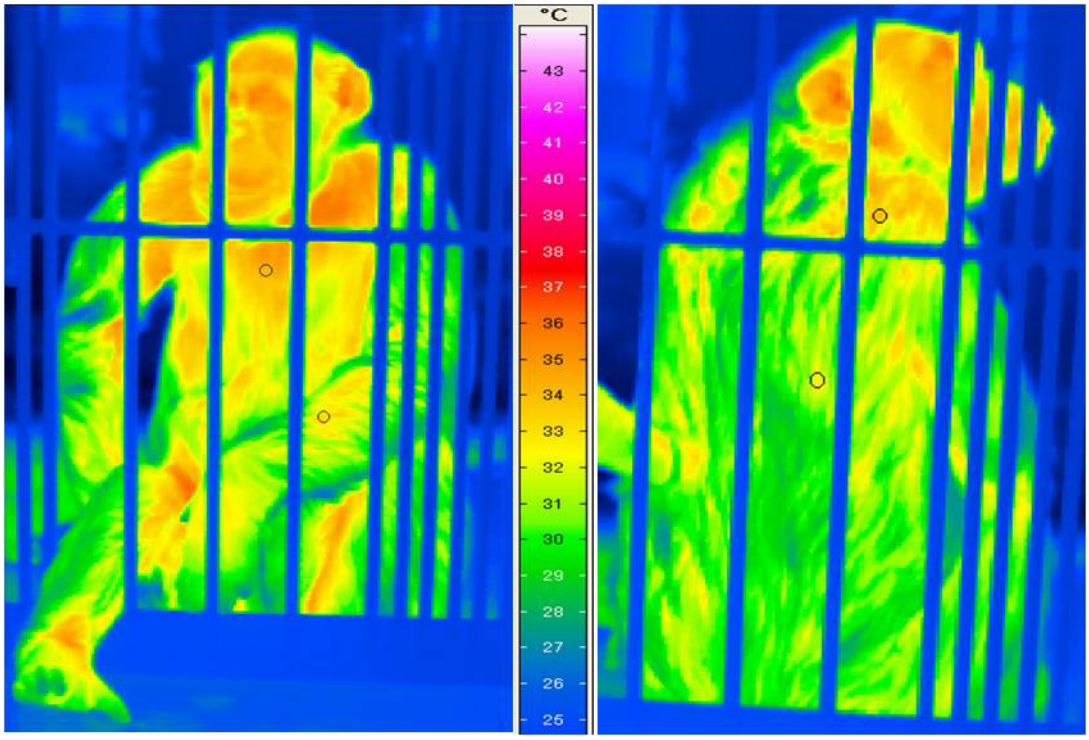

Figure 3.5: Thermal images (frontal view and back view) with temperature scale in °C from one female zoo-living chimpanzee. Black circles indicate the locations from which temperature measure were extracted.

3.5 Discussion

Hair cortisol analysis is increasingly applied to measure long-term stress in animals and humans, but we still lack sufficient understanding of potentially confounding effect, such as the waning effect along the hair shaft and possible differences in steroid incorporation at different body regions. In this study, we estimated these effects using chimpanzee hairs.

Concerning the waning effect, our results revealed a strong HCC decrease towards the distal end of the hair shaft in samples from Ngamba Island chimpanzees as well as a significant though very weak effect in samples from European zoo chimpanzees. While this is in line with many human studies (Dettenborn et al., 2012; Kirschbaum et al., 2009; Skoluda et al., 2012, but see Manenschijn et al., 2011; Thomson et al., 2010), the present results oppose all previous animal studies, which reported no systematic cortisol decrease towards distal segments (Bennett and Hayssen, 2010; Carlitz et al., 2014; Davenport et al., 2006; Macbeth et al., 2010; Yamanashi et al., 2013).

It is unlikely that differences in laboratory protocols, as suggested by Manenschijn et al. (2011), led to the discrepancy between the present and former results because the same protocol was also applied in our orangutan study, in which no cortisol decrease along 15 cm long hair shafts was observed (Carlitz et al., 2014). It is also unlikely that this is a species specific result since Yamanashi et al. (2013) did not find a waning effect in chimpanzees either. There might be a difference between captive animals that are sheltered from ambient weather conditions and (semi-) wild animals that are exposed to ambient weathers. However, Macbeth et al. (2010), the only study investigating HCC along the hair shaft in wild animals, did not find a waning effect either. Yet, it is conceivable that the formerly applied statistical methods (repeated-measures ANOVA) were not sensitive enough because it pools valuable information. Interestingly, a repeated-measures ANOVA would not have brought to light the small waning effect in the present samples from zoo chimpanzees but only in the NI samples (data not shown). The detailed graph of cortisol changes along the hair shaft (Figure 3.2) illustrates that in zoo samples the effect is masked by considerable background noise and is only visible in the second and third segment if samples with greater initial HCC are included. This may be due to the fact that individual samples with low (initial) HCC are more likely to show an increase or no change over time rather than a decrease of HCC due to bottom effects. Furthermore, it is conceivable that small quantities of cortisol exhibit stronger bonds to the hair matrix and are less likely to be removed by external factors than larger quantities of cortisol. Macbeth et al. (2010) already mentioned the possibility that a waning effect was not detectable in their study on wild grizzly bears because of low HCC in their samples.

In search of underlying mechanisms for the systematic cortisol decrease observed in the present study, internal factors (i.e., stress related) as well as external factors (e.g., rain, sun) have to be considered. Concerning internal factors, it seems unlikely that stress levels decreasing systematically across the time period covered by the hair segments affected the whole NI group in two consecutive years and the inhomogeneous zoo group alike, resulting in systematic cortisol decrease along the hair shaft. Concerning external factors, water (hair washes in humans or rain in animals) has often been suggested as the primary source of cortisol decrease along the hair shaft, which is why this effect was called washout effect (Kirschbaum et al., 2009). To date, however, only two *in vitro* studies (Hamel et al., 2011; Li et al., 2012), revealed a direct relationship between exposure to water and HCC decrease along the hair. Moreover, the one *in vivo* study (Dettenborn et al., 2012), which tried to correlate the number of weekly hair washes in humans with the waning effect along the hair shaft, may not have found the effect because of too many other factors that add noise to the data.

The *in vitro* experiment from Li et al. (2012) suggests that water and UV-irradiation can reduce HCC independently, although *in vivo* data for the effect of UV-irradiation on HCC is still pending. The pronounced cortisol decrease in NI samples and the very weak effect in samples from zoo animals could also be interpreted as the result of differences in exposition to UV-irradiation (moderate European vs. strong equatorial sun), difference in exposition to water (negligible in European zoos vs. routinely in NI animals), or differences of a combination of both. However, this interpretation at present remains speculative because of the higher noise level (i.e., fluctuation of the environment) in the inhomogeneous zoo samples, and a weakened hair structure from one factor with cortisol removal, or degradation from another factor (*cf.* Manenschijn et al., 2011) can also not be excluded. Thus, the underlying mechanisms for the waning effect remain to be investigated as well as the question to what extent hair samples from the European zoo animals are affected.

Despite the presence of the waning effect our results showed a strong intra-sample stability in both groups and all segments provided similar biological information (although absolute HCC differed between segments). Thus, if animals are exposed to similar ambient conditions, the effect can be ‘controlled for’ using the same length of hair throughout the study.

Concerning the body-region effect, our results revealed that absolute HCC differed significantly between body regions, which is in line with previous studies (Macbeth et al., 2010; Moya et al., 2013; Terwissen et al., 2013; Yamanashi et al., 2013). In addition, and similar to the chimpanzee study from Yamanashi et al. (2013), our results revealed strong correlations between body regions. The present data extend previous research showing that HCC measures of all body regions were mainly driven by one common factor. Thus, HCC in all body regions appear to convey information about the same biological entity, and it is likely that this biological entity is an excellent representation of the systemic cortisol secretion as suggested by an increasing body of literature in animals (e.g., Carlitz et al., 2014; del Rosario Gonzalez-de-la-Vara et al., 2011; Malcolm et al., 2013; Mastromonaco et al., 2014; Terwissen et al., 2013) and humans (e.g., O'Brien et al., 2013; Stalder et al., 2014). Assuming no asymmetry of hair samples from different regions, controlling for body region could be omitted if a large enough sample from random body regions is available, even though it inevitably will reduce the signal-to-noise ratio. Thus, it is possible to use shed hair samples as a mixture of various body regions.

In an attempt to explain the underlying mechanism of the effect of body region, we found that skin temperature, which is a measure of skin blood flow (Rubinstein and Sessler, 1990), increased in the same order as the mean HCC levels from the 38 NI chimpanzees (back<forearm<shoulder<chest). Even though there is an unexplained mismatch for one body region between skin temperature and HCC in the particular subject for which skin temperature was recorded, it is conceivable that higher skin blood flow indicates a greater blood supply to the hair root per unit time, which might result in a higher diffusion rate and therefore in more cortisol incorporation into the hair shaft. Indeed, HCC patterns of body regions described earlier for chimpanzees (side > back > elbow; Yamanashi et al., 2013) and for orang-utans (shoulder > stomach = back > wrist, although this pattern was not significant; Carlitz et al., 2014) also seem to follow the skin temperature gradient illustrated in the thermal images of one chimpanzee (Figure 3.5). While these results encourage further investigations of the relationship between HCC and skin blood flow, more data with temperature measures from more animals as well as from more body regions are necessary to verify this skin blood flow hypothesis. Yet, future work should also consider a potential interaction between the systemic and local cortisol production as suggested

by Keckeis et al. (2012), although Pang et al. (2014) found that local cortisol production in the skin was reduced with increased systemic cortisol concentration.

In conclusion, this study presents first evidence that the waning effect, so far only reported for human hair, influences HCC along the hair shaft of chimpanzees. Nonetheless, all segments provided similar biological information. Regarding the body-region effect, our results confirm that absolute HCC differ between body regions and extend previous research by showing that HCC in all body regions convey similar biological information, presumably the systemic cortisol secretion. In conclusion, shed hair from various unknown body regions can well be used in observational studies at the cost of a lower signal-to-noise ratio.

3.6 Acknowledgements

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4. Subjective stress estimates from animal keepers strongly correlate with physiological stress measures obtained from hair cortisol concentrations in captive chimpanzees (*Pan troglodytes*)

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Submitted manuscript

4.1 Abstract

Subjective estimates from animal keepers on the animals' well-being are commonly used for decisions on captive management in zoos and sanctuaries because keeper-derived estimates are suggested to predict animal well-being most reliably due to their close relationship with the animals. However, the extent to which subjective judging can reflect physiological stress is unclear. The present study set out to examine how well animal keeper-derived stress estimates correlate with physiological stress measures in 36 semi-wild sanctuary chimpanzees (*Pan troglodytes*), as determined by cortisol concentrations in hair (HCC). In preparatory analyses testing for covariates, significantly higher HCC was found in males than in females [$\chi^2(1) = 10.9$, $p < 0.001$, 28% explained variance] whereas age was unrelated to HCC [$\chi^2(2) = 0.34$, $p = 0.84$]. A regression model accounting for sex differences revealed that keeper-derived stress rankings of chimpanzees explained 52% of the variance in the HCC measure [$\chi^2(1) = 63.7$, $p < 0.001$] and partial Spearman rank correlation showed a large positive association between keeper-derived stress rankings and HCC ($pr_s = 0.53$, $p < 0.001$). Thus, well-trained animal

[Please, insert article 1 here.]

5. Monitoring long-term stress through hair cortisol analysis to assess the effect of anthropogenic impacts on wild chimpanzees (*Pan troglodytes*)

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Submitted manuscript

5.1 Abstract

Non-human primates face major environmental changes due to increased human impacts all over the world. Although some species are able to survive in certain landscapes with anthropogenic impact, their long-term viability and fitness may be decreased due to chronic stress. Here we assessed long-term stress levels through cortisol analysis in chimpanzee hair obtained from sleeping nests in northwestern Uganda, in order to estimate welfare in the context of ecotourism, forest fragmentation with human-wildlife conflicts, and illegal logging with hunting activity (albeit not of primates), compared with a control without human contact or conflict. Concerning methodological issues, season (r^2 = 0.18) and the age of nests (r^2 = 0.11) significantly predicted hair cortisol levels. With regard to effects of anthropogenic impacts, our results showed no elevation of hair cortisol concentrations (HCC) due to ecotourism, nor due to illegal logging compared to their control groups. We did, however, find significantly increased HCC in the fragment group compared to chimpanzees living in a nearby intact forest (r^2 = 0.20). In conclusion, our results suggest that hair cortisol analysis is a powerful tool that can help understanding the impact of anthropogenic disturbances on chimpanzee well-being and could be easily applied to other great ape species.

[Please, insert article 2 here.]

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Curriculum Vitae

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